α-NAPHTHOL A PRECURSOR OF VITAMIN K_2

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Relatively little is known of the biosynthesis of the naphthoquinone nucleus in microorganisms and higher plants. Cox and Gibson (1964, 1966) have demonstrated the incorporation of shikimic acid (U-^{14}C) into vitamin K_2 (4o) in E. coli. Degradation of the labelled vitamin K showed that shikimic acid is incorporated into the benzene ring of this naphthoquinone. 3,4-Dihydroxybenzaldehyde was also implied by these authors as a possible intermediate in the biosynthesis of this vitamin. We now report the incorporation of α-naphthol into the naphthoquinone nucleus of vitamin K_2 in whole cells of Bacillus megaterium which indicates this compound to be a precursor of vitamin K_2 in microorganisms.

METHODS: B. megaterium cells were grown aerobically in 1 liter of synthetic medium (glycerol-citrate-mineral salts medium containing 10^{-4} M L-phenylalanine, L-tyrosine and L-tryptophan) for 24 hrs at 36°C in the presence of labelled precursors which had been sterilized separately. The cells (wet weight ca. 6 g) were extracted by the methanol method. Vitamin K was isolated and determined according to Bishop et al. (1962). The presence of vitamin K_2 (35) in B. megaterium which was reported by these authors was confirmed by chromatography along with the authentic

compound. The vitamin K_2 (35) in the hexane extract was purified by repeated thin layer chromatography on Kieselgel GF_{254} (E.Merck) (solvent system benzene: petroleum ether (30-70°) = 3:1) and by running the sample on reversed phase thinlayer (acetone : H_2O = 95 :5) until constant specific activity was reached. After dilution (1:10) with non-radioactive vitamin K_2 (20) the labelled compound (about 10 µmole) was degraded to phthalic acid by refluxing it with $KMnO_4$ (30 mg) in acetone (1 ml) for 1,5 hours. Phthalic acid was purified and decarboxylated as described previously (Leistner and Zenk, 1967). α-Naphthol (1-^{14}C) was purchased from the Radiochemical Center, shikimic acid (U-^{14}C) from New England Nuclear Corp., shikimic acid (1,2-^{14}C) and veratric acid (7-^{14}C) from Commissariat a l'Energie Atomique. 3,4-Dihydroxybenzoic acid (7-^{14}C) was prepared from veratric acid (Zenk, 1965) and protocatechualdehyde was tritiated by Dr.H.G.Floss, Munich. The radioactivity was measured after combustion of the samples by the Schöninger method (Kalberer and Rutschmann, 1961) with a Nuclear Chicago Unilux Scintillationspectrometer.

RESULTS AND DISCUSSION: Incorporation experiments with 6 different species of bacteria (*E. coli*, *Bacillus subtilis*, *Proteus vulgaris*, *Sarcina lutea*, *Micrococcus lysodeicticus*, *B. megaterium*) showed, that the highest incorporation of shikimic acid (^{14}C) into vitamin K_2 was obtained with *B. megaterium*. This organism, therefore, was used for further experiments. The results of the feeding experiments with different potential precursors are shown in Table I. It is clear from these results that shikimic acid is an efficient precursor of this naphthoquinone. Also α-naphthol is incorporated well. α-Naphthol, which should be a closer precursor to the

naphthoquinone than shikimic acid, is incorporated only to an equal extend. This is probably due to penetration difficulties and to the fact that the phenolic hydroxyl group of α-naphthol is readily glucosylated.

TABLE I: Incorporation of different potential precursors into vitamin K_2 (35) of B. megaterium.

Precursor				Vitamin K_2 (35)		
name	µmole fed	dpm $\times 10^6$	dpm$\times 10^6$ in cells	µmole isolated	spec. activity (dpm/µmole)	incorporation (%)
DL-Shikimic acid (1,2-^{14}C)	0,26	6,00	1,02	0,81	33 400	2,65
D-Shikimic acid (U-^{14}C)	0,50	3,75	2,46	1,42	19 750	1,14
α-Naphthol (1-^{14}C)	0,30	11,10	0,88	1,10	11 960	1,50
3,4-Dihydroxy-benzaldehyde (G-^{3}H)	31,50	101,00	0,15	0,98	0	0
3,4-Dihydroxy-benzoic acid (7-^{14}C)	2,23	3,22	0,08	1,17	0	0

It was surprising however to find that 3,4-dihydroxybenzaldehyde is not incorporated into vitamin K_2. This result has been confirmed with E.coli and casts some doubt on the possible precursor function of this compound as postulated by Cox and Gibson (1964, 1966). Concomitantly an experiment was conducted with E. coli by supplying shikimic acid (1,2-^{14}C) in the presence and absence of 10^{-4} M 3,4-dihydroxybenzaldehyde. In the presence of the aldehyde

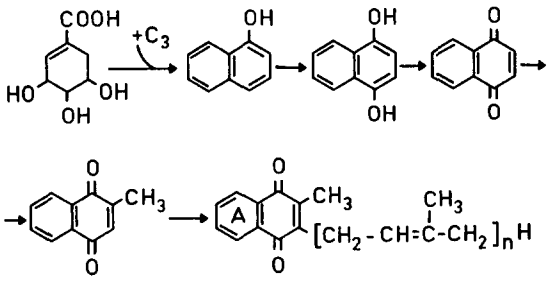
the incorporation of shikimic acid into ubiquinone was not affected at all, while the incorporation into vitamin K_2 was suppressed by 4o %. However, neither labelled 3,4-dihydroxybenzaldehyde nor 3,4-dihydroxybenzoic acid, a "sixth factor" of Davis (1952), was incorporated under these conditions. This indicates that the effect of 3,4-dihydroxybenzaldehyde on vitamin K biosynthesis must be an indirect one and cannot be explained with a precursor function of this compound. 3,4-Dihydroxybenzaldehyde-^{3}H has been recovered unchanged from the incubation medium. This tritiated compound had successfully been employed in precursor feeding experiments (alkaloid biosynthesis) without exchange and loss of tritium by Suhadolnik et al.(1962).

Degradation of labelled vitamin K_2 showed that shikimic acid and α-naphthol are incorporated without randomisation. Furthermore Table II definitely confirms the report of Cox and Gibson (1966) that the ring atoms of shikimic acid are incorporated into the benzene ring (A) of the naphthoquinone.

TABLE II: Degradation of labelled vitamin K_2 (35)

	Vit.K_2	Phthalic acid		CO_2	
	dpm/ mmole·10^{-6}	dpm/ mmole· 10^{-6}	% of Vit.K_2	dpm/ mmole· 10^{-6}	2 CO_2 % of Phthalic acid
DL-Shikimic acid (1,2-^{14}C)	1,94	1,97	1o2	o	o
D-Shikimic acid (U-^{14}C)	9,52	9,48	99,6	o,66	13,9
α-Naphthol (1-^{14}C)	o,5o	o,51	1o2	o,26	1o2

This is in absolute disagreement with the results obtained by Chen and Bohm (1966) and Bohm (1967) who claim that shikimic acid is incorporated into the quinone ring during naphthoquinone biosynthesis in plants. In addition our results show for the first time that shikimic acid is incorporated *in toto* during vitamin K biosynthesis, the carboxylgroup (15 % ^{14}C) of the uniformly labelled acid being transformed into one, or equally into both of the keto groups (C-1 and -4) of the quinone ring (found: 13,9 % ^{14}C). α-Naphthol is also a precursor and its radioactive C-1-atom is transformed into the ketogroups of the naphthoquinone molecule which are represented by the carboxylgroups of phthalic acid after oxidation of vitamin K_2. The observed incorporation of α-naphthol into vitamin K proves the correctness of the hypothesis that α-naphthol is an early precursor of plant naphthoquinones conceived purely on comparative phytochemical grounds by Sandermann and Simatupang (1966, 1967). All these data together with the experiments of Martius and Leuzinger (1964) suggest the following incomplete biosynthetic sequence for the formation of vitamin K :



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